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(54) Title: POLYNUCLEOTIDE COMPOSITIONS

(57) Abstract

The invention provides compositions for stabilizing polynucleic acids and increasing the ability of polynucleic acids to cross cell membranes and act in the interior of a cell. In one aspect, the invention provides a polynucleotide complex between a polynucleotide and certain polyether block copolymers. Preferably the polynucleotide complex will further include a polycationic polymer. In another aspect, the invention provides a polynucleotide complex between a polynucleotide and a block copolymer comprising a polyether block and a polycation block. In yet another aspect, the invention provides polynucleotides that have been covalently modified at their 5' or 3' end to attach a polyether polymer segment. In still another aspect, the invention provides certain preferred polycationic polymers.

POLYNUCLEOTIDE COMPOSITIONS

This application is a continuation-in-part of U.S. Application No. 08/342,209, filed November 18, 1994, titled "Polynucleotide Compositions", and is incorporated herein by reference.

The present invention relates to compositions of poly(nucleic acid) polymers such as RNA or DNA polymers and polycations that are associated, either covalently or noncovalently, with block copolymers of alkylethers. In a preferred embodiment, the poly(nucleic acids) will be complexed with a polycation. The poly(nucleic acid) is stabilized by the complex and, in the complex, has increased permeability across cell membranes. Accordingly, the complexes are well suited for use as vehicles for delivering nucleic acid into cells.

The use of "antisense" poly(nucleic acid) to treat genetic diseases, cell mutations (including cancer causing or enhancing mutations) and viral infections has gained widespread attention. This treatment tool is believed to operate, in one aspect, by binding to "sense" strands of mRNA encoding a protein believed to be involved in causing the disease state sought to be treated, thereby stopping or inhibiting the translation of the mRNA into the unwanted protein. In another aspect, genomic DNA is targeted for binding by the antisense polynucleotide (forming a triple helix), for instance, to inhibit transcription. See, Helene, *Anti-Cancer Drug Design*, 6:569 (1991). Once the sequence of the mRNA sought to be bound is known, an antisense molecule can be designed that binds the sense strand by the Watson-Crick base-pairing rules, forming a duplex structure analogous to the DNA double helix. *Gene Regulation: Biology of Antisense RNA and DNA*, Erikson and Ixzant, eds., Raven Press, New York, 1991; Helene, *Anti-Cancer Drug Design*, 6:569 (1991); Crooke, *Anti-Cancer Drug Design*, 6:609 (1991). A serious barrier to fully exploiting this technology is the problem of efficiently introducing into cells a sufficient number of antisense molecules to effectively interfere with the translation of the targeted mRNA or the function of DNA.

One method that has been employed to overcome this problem is to covalently modify the 5' or the 3' end of the antisense poly(nucleic acid) molecule with hydrophobic substituents. These modified nucleic acids generally

an organism, a DNA vector capable of directing the synthesis of a protein missing from the cell or useful to the cell or organism when expressed in great r amounts. The methods for introducing DNA to cause a cell to produce a new protein, ribozyme or a greater amount of a protein or ribozyme are called "transfection" methods. See, generally, *Neoplastic Diseases*, Huber and Lazo, eds., New York Academy of Science, New York, 1994; Feigner, *Adv. Drug Deliv. Rev.*, 5:163 (1990); McLachlin, et al., *Progr. Nucl. Acids Res. Mol. Biol.*, 38:91 (1990); Karlsson, *S. Blood*, 78:2481 (1991); Einerhand and Valerio, *Curr. Top. Microbiol. Immunol.*, 177:217-235 (1992); Makdisi et al., *Prog. Liver Dis.*, 10:1 (1992); Litzinger and Huang, *Biochim. Biophys. Acta*, 1113:201 (1992); Morsy et al., *J.A.M.A.*, 270:2338 (1993); Dorudi et al., *British J. Surgery*, 80:566 (1993).

A number of the above-discussed methods of enhancing cell penetration by antisense poly(nucleic acid) are generally applicable methods of incorporating a variety of poly(nucleic acids) into cells. Other general methods include calcium phosphate precipitation of poly(nucleic acid) and incubation with the target cells (Graham and Van der Eb, *Virology*, 52:456, 1983), co-incubation of poly(nucleic acid), DEAE-dextran and cells (Sompayrac and Danna, *Proc. Natl. Acad. Sci.*, 12:7575, 1981), electroporation of cells in the presence of poly(nucleic acid) (Potter et al., *Proc. Natl. Acad. Sci.*, 81:7161-7165, 1984), incorporating nucleic acid into virus coats to create transfection vehicles (Gitman et al., *Proc. Natl. Acad. Sci. U.S.A.*, 82:7309-7313, 1985) and incubating cells with poly(nucleic acid) incorporated into liposomes (Wang and Huang, *Proc. Natl. Acad. Sci.*, 84:7851-7855, 1987).

Another problem in delivering poly(nucleic acid) to a cell is the extreme sensitivity of poly(nucleic acids), particularly ribonucleic acids, to nuclease activity. This problem has been particularly germane to efforts to use ribonucleic acids as anti-sense oligonucleotides. Accordingly, methods of protecting poly(nucleic acid) from nuclease activity are desirable.

SUMMARY OF THE INVENTION

The invention is described below with reference to the fragmental constants developed by Hansch and Leo. See Hansch and Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley*, New York,

inserting poly(nucleic acid) into cells utilizing the first embodiment polynucleotide composition of the invention.

In a second embodiment, the invention provides a polynucleotide composition comprising:

(a) a polynucleotide or derivative;

(b) a block copolymer having a polyether segment and a polycation segment, wherein the polyether segment comprises at least an A-type block, and the polycation segment comprises a plurality of cationic repeating units.

In a preferred embodiment, the block copolymer further comprises a B-type block. In another preferred second embodiment, the copolymer comprises a polymer of formula:

B-A-R,	**A-R,**	**A-R-A',**	and	**R-A-R',**
(V-a)	(VI-a)	(VII)		(VIII-a)
	A-B-R,	**A-R-B,**	**R-A-B**	
(V-b)	(VI-b)	(VIII-b)		

wherein the A, A' and B are as described above, wherein R and R' are polymeric segments comprising a plurality of cationic repeating units, wherein each cationic repeating unit in a segment may be the same or different from another unit in the segment. The polymers of this embodiment can be termed "polyether/polycation" polymers. The R and R', blocks can be termed "R-type" polymeric segments or blocks.

The polynucleotide composition of the second embodiment provides an efficient vehicle for introducing the polynucleotide into a cell. Accordingly, the invention also relates to a method of inserting poly(nucleic acid) into cells utilizing the second embodiment composition of the invention.

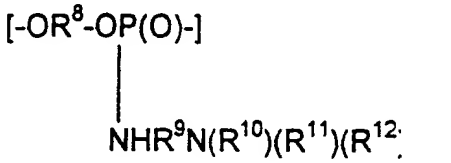
In a third embodiment, the invention provides a polynucleotide composition comprising a polynucleotide derivative comprising a polynucleotide segment and a polyether segment attached to one or both of the polynucleotide 5' and 3' ends, wherein the polyether comprises an A-type polyether segment. In a preferred third embodiment, the derivative comprises at least three blocks, at least two of which are A-type or B-type blocks. In another preferred third embodiment, the derivative comprises a block copolymer of formulas:

B-A-R, A-R, A-R-A', and R-A-R',

(V) (VI) (VII) (VIII)

wherein A, A' and B are as described above, wherein R and R' are polymeric segments comprising a plurality of cationic repeating units of formula -NH-R^0-, wherein R^0 is a straight chain aliphatic group having from 2 to 6 carbon atoms, which may be substituted. Each -NH-R^0- repeating unit in an R-type segment can be the same or different from another -NH-R^0- repeating unit in the segment. A preferred fourth embodiment further comprises a polynucleotide or derivative.

In a fifth embodiment, the invention provides a polycationic polymer comprising a plurality of repeating units of formula:



where R^8 is:

(1) -$(CH_2)_n$-$CH(R^{13})$-, wherein n is an integer from 0 to about 5 and R^{13} is hydrogen, cycloalkyl having 3-8 carbon atoms, alkyl having 1-6 carbon atoms, or $(CH_2)_mR^{14}$, where m is an in[illegible] 0 to about 12 and R^{14} is a lipophilic substituent of 6 to [illegible] atoms;

(2) a carbocyclic group having 3-8 ring carbon atoms, wherei[illegible] group can be for example, cycloalkyl or aromatic groups, and whic[illegible] include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon ato[illegible], alkylamino having 1-6 carbon atoms, dialkylamino wherein each alkyl independently has 1-6 carbon atoms, amino, sulfonyl, hydroxy, carboxy, fluoro or chloro substituents; or

(3) a heterocyclic group, having 3-8 ring atoms, which can include heterocycloalkyl or heteroaromatic groups, which can include from 1 to 4 heteroatoms selected from the group consisting of oxygen, nitrogen, sulfur and mixtures thereto, and which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino having 1-6 carbon

polymerization shall be between about 5 and about 180, still more preferably, between about 5 and about 60. The degree of polymerization of the polycationic polymer can preferably be between about 10 and about 10,000. More preferably, the degree of polymerization shall be between about 10 and about 1,000, still more preferably, between about 10 and about 100.

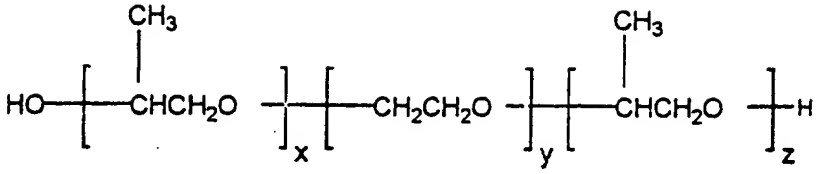
The repeating units that comprise the blocks, for A-type, B-type and R-type blocks, will generally have molecular weight between about 30 and about 500, preferably between about 30 and about 100, still more preferably between about 30 and about 60. Generally, in each of the A-type or B-type blocks, at least about 80% of the linkages between repeating units will be ether linkages, preferably, at least about 90% will be ether linkages, more preferably, at least about 95% will be ether linkages. Ether linkages, for the purposes of this application, encompass glycosidic linkages (i.e., sugar linkages). However, in one aspect, simple ether linkages are preferred.

Preferably, all of the repeating units that comprise A-type blocks have a Hansch-Leo fragmental constant of less than about -0.4, more preferably, less than about -0.5, still more preferably, less than about -0.7. Preferably, all of the repeating units that comprise B-type blocks have a Hansch-Leo fragmental constant of about -0.30 or more, more preferably about -0.20 or more.

The polynucleotide component (pN) of formulas (IX) through (XIII) will preferably comprise from about 5 to about 1,000,000 bases, more preferably about 5 to about 100,000 bases, yet more preferably about 10 to about 10,000 bases.

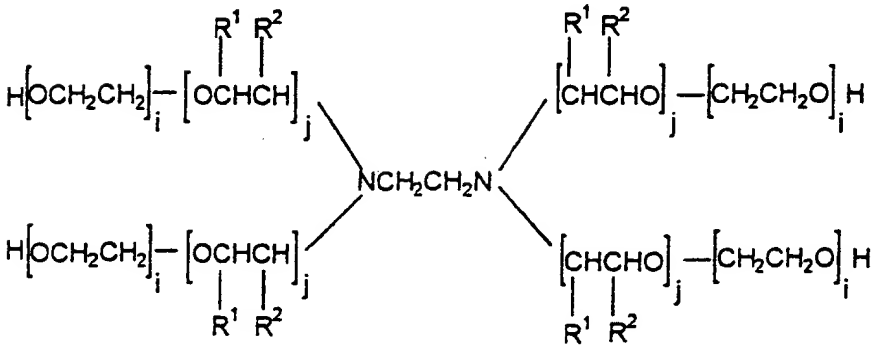
The polycationic polymers and the R-type blocks have several positively ionizable groups and a net positive charge at physiologic pH. The polyether/polycation polymers of formulas (V) - (VIII) can also serve as polycationic polymers. Preferably, the polycationic polymers and R-type blocks will have at least about 3 positive charges at physiologic pH, more preferably, at least about 6, still more preferably, at least about 12. Also preferred, are polymers or blocks that, at physiologic pH, can present positive charges with about a spacing between the charges of about 3 Å to about 10 Å . The spacings established by aminopropylene repeating units, or by mixtures of aminopropylene and

or,



(XVI)

or,



(XVII)

in which x, y, z, i and j have values from about 5 to about 400, preferably from about 5 to about 200, more preferably from about 5 to about 80, and wherein for each R^1, R^2 pair, one shall be hydrogen and the other shall be a methyl group. Formulas (XIV) through (XVI) are oversimplified in that, in practice, the orientation of the isopropylene radicals within the B block will be random. This random orientation is indicated in formula (XVII), which is more complete. Such poly(oxyethylene)-poly(oxypropylene) compounds have been described by Santon, *Am. Perfumer Cosmet.* 72(4):54-58 (1958); Schmolka, *Loc. cit.* 82(7):25-30 (1967); *Non-ionic Surfactants*, Schick, ed. (Dekker, NY, 1967), pp. 300-371. A number of such compounds are commercially available under such generic trade names as "poloxamers," "pluronics" and "synperonics." Pluronic polymers within the B-A-B formula are often referred to as "reversed" pluronics, "pluronic R" or

Thus, the units making up the first block need not consist solely of ethylene oxide. Similarly, not all of the B-type block need consist solely of propylene oxide units. Instead, the blocks can incorporate monomers other than those defined in formulas (XIV)-(XVII), so long as the parameters of the first embodiment are maintained. Thus, in the simplest of examples, at least one of the monomers in block A might be substituted with a side chain group as previously described.

In another aspect, the invention relates to a polynucleotide complex comprising a block copolymer at least one of formulas (I) - (XIII), wherein the A-type and B-type blocks are substantially made up of repeating units of formula -O-R^5, where R^5 is:

(1) $-(CH_2)_n-CH(R^6)-$, wherein n is an integer from 0 to about 5 and R^6 is hydrogen, cycloalkyl having 3-8 carbon atoms, alkyl having 1-6 carbon atoms, phenyl, alkylphenyl wherein the alkyl has 1-6 carbon atoms, hydroxy, hydroxyalkyl, wherein the alkyl has 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, an alkyl carbonyl group having 2-7 carbon atoms, alkoxycarbonyl, wherein the alkoxy has 1-6 carbon atoms, alkoxycarbonylalkyl, wherein the alkoxy and alkyl each independently has 1-6 carbon atoms, alkylcarboxyalkyl, wherein each alkyl group has 1-6 carbon atoms, aminoalkyl wherein the alkyl group has 1-6 carbon atoms, alkylamine or dialkylamino, wherein each alkyl independently has 1-6 carbon atoms, mono- or di-alkylaminoalkyl wherein each alkyl independently has 1-6 carbon atoms, chloro, chloroalkyl wherein the alkyl has from 1-6 carbon atoms, fluoro, fluoroalkyl wherein the alkyl has from 1-6 carbon atoms, cyano or cyano alkyl wherein the alkyl has from 1-6 carbon atoms or carboxyl;

(2) a carbocyclic group having 3-8 ring carbon atoms, wherein the group can be for example, cycloalkyl or aromatic groups, and which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino having 1-6 carbon atoms, dialkylamino wherein each alkyl

$$2f_C + 4f_H + f_O + (4-1)F_b = 2(0.20) + 4(0.23) + (-1.82) + 3(-0.12) = -0.86$$

The Hansch-Leo fragmental constant for a propylene oxide ($-CH_2CHCH_3O-$) repeating unit would be:

$$2f_C + f_{CH3} + 3f_H + f_O + (4-1)F_b = 2(0.2) + 0.89 + 3(0.23) + (-1.82) + 3(-0.12) = -0.2$$

Those of ordinary skill in the art will recognize that the Hansch-Leo approach to estimating partition constants, in which approach the Hansch-Leo fragmental constants are applied, does not yield precisely the empirical partition constant. See Hansch and Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley*, New York, 1979; James, *Solubility and Related Properties*, Marcel Dekker, New York, 1986, pp. 320-325. However, the approach is precise enough to define the hydrophobicity features of the polymeric delivery vehicle.

A wide variety of poly(nucleic acid) molecules can be the poly(nucleic acid) component of the composition. These include natural and synthetic DNA or RNA molecules and nucleic acid molecules that have been covalently modified (to incorporate groups including lipophilic groups, photo-induced crosslinking groups, alkylating groups, organometallic groups, intercalating groups, lipophilic groups, biotin, fluorescent and radioactive groups, and groups that modify the phosphate backbone). Such nucleic acid molecules can be, among other things, antisense nucleic acid molecules, gene-encoding DNA (usually including an appropriate promotor sequence), ribozymes oligonucleotide α-anomers, ethylphosphotriester analogs, alkylphosphomates, phosphorothionate and phosphorodithionate oligonucleotides, and the like . In fact, the nucleic acid component can be any nucleic acid that can beneficially be transported into a cell with greater efficiency, or stabilized from degradative processes, or improved in its biodistribution after administration to an animal.

Examples of useful polymers pursuant to formulas (V) - (VIII) include the poly(oxyethylene)-poly-L-lysine) diblock copolymer of the following formula:

$$CH_3O\text{-}(CH_2CH_2O)_i CO[(NH(CH_2)_3)_2NH(CH_2)_4]_j\text{-}(NH(CH_2)_3)_2\text{-}NHCO\text{-}O\text{-}(CH_2CH_2O)_k\text{-}CH_3$$

(XXII)

wherein i is an integer from about 10 to about 200, j is an integer from about 1 to about 8, and k is an integer from about 10 to about 200. Still another example is the polymer of formula:

$$H\text{-}G_j\text{-}(NH(CH_2)_3)_2\text{-}N\text{-}NH\text{-}CO\text{-}O\text{-}(CH_2CH_2O)_i CO\text{-}G_m\text{-}(NH(CH_2)_3)_2\text{-}NH_2$$

(XXIII)

wherein "G" comprises $-(NH(CH_2)_3)_3\text{-}CH_2NH_2-$, i and j are as defined for formula (XVIII), and m is an integer from about 1 to about 8.

The block copolymers utilized in the invention, will typically, under certain circumstances, form micelles of from about 10 nm to about 100 nm in diameter. Micelles are supramolecular complexes of certain amphiphilic molecules that form in aqueous solutions due to microphase separation of the nonpolar portions of the amphiphiles. Micelles form when the concentration of the amphiphile reaches, for a given temperature, a critical micellar concentration ("CMC") that is characteristic of the amphiphile. Such micelles will generally include from about 10 to about 300 block copolymers. By varying the sizes of the hydrophilic and hydrophobic portions of the block copolymers, the tendency of the copolymers to form micelles at physiological conditions can be varied. The micelles have a dense core formed by the water insoluble repeating units of the B blocks and charge-neutralized nucleic acids, and a hydrophilic shell formed by the A blocks. The micelles have translational and rotational freedom in solution, and solutions containing the micelles have low viscosity similar to water. Micelle formation typically occurs at copolymer concentrations from about 0.001 to 5% (w/v). Generally, the concentration of polycationic polymers and polynucleic acid will be less than the concentration of copolymers in the polynucleotide compositions, preferably at least about 10-fold less, more preferably at least about 50-fold.

At high concentrations, some of the block copolymers utilized in the invention will form gels. These gels are viscous systems in which the translational and rotational freedom of the copolymer molecules is significantly constrained by a continuous network of interactions among copolymer molecules. In gels, microsegregation of the B block repeating units may or may

preferably ϕ will range between about 0.01 and about 50, more preferably, between about 0.1 and about 20. ϕ can be varied to increase the efficiency of transmembrane transport and, when th composition comprises polynucleotide complexes, to increase the stability of the complex. Variations in ϕ can also affect the biodistribution of the complex after administration to an animal. The optimal ϕ will depend on, among other things, (1) the context in which the polynucleotide composition is being used, (2) the specific polymers and oligonucleotides being used, (3) the cells or tissues targeted, and (4) the mode of administration.

In some preferred embodiments the ability of the conjugate to form a micelle is believed to correlate with certain desirable characteristics such as ability to be solubilized in aqueous and non-aqueous media, which solubility characteristic facilitates transmembrane transport. Micelle-forming ability of a block copolymer is believed to correlate with the presence of hydrophobic and hydrophilic polymeric blocks. Hydrophobic blocks are provided by B-type blocks and polynucleotide segments that are charge-neutralized with polycationic polymers, R-type blocks or certain hydrophobic, non-polymer cations. Hydrophilic blocks are provided by A-type blocks and, to some extent, polynucleotide segments that are incompletely neutralized by ionic species that confer hydrophobicity. The block copolymers of the invention preferably include an A-type block which serves to increase solubility and decrease interactions with non-target molecules and cells.

It will in some circumstances be desirable to incorporate, by noncovalent association, targeting molecules. See, for example, Kabanov et al., *J. Controlled Release*, 22:141 (1992). The targeting molecules that can be associated with the composition typically have a targeting group having affinity for a cellular site and a hydrophobic group. The targeting molecule will spontaneously associate with the polynucleotide complex and be "anchored" thereto through the hydrophobic group. These targeting adducts will typically comprise about 10% or less of the copolymers in a composition.

In the targeting molecule, the hydrophobic group can be, among other things, a lipid group such as a fatty acyl group. Alternately, it can be a block

A polynucleotide functions include one or more of the following: binding to another polynucleotide being effect to transfect, being repressed, directing the synthesis of a protein, incorporation into a RNA or DNA or genome, acting as a ribozyme and the like.

For polyethylene oxide-polypropylene oxide copolymer, the hydrophilic/hydrophobic properties, and micelle forming properties of a block copolymer are, to a certain degree, related to the value of the ratio, n. The ratio, n, is defined as:

$$n = (|B|/|A|) \times (b/a) = (|B|/|A|) \times 1.32$$

where |B| and |A| are the number of repeating units in the hydrophobic and hydrophilic blocks of the copolymer, respectively, and **b** and **a** are the molecular weights for the respective repeating units. The value of n will typically be between about 0.2 and about 9.0, more preferably, between about 0.2 and about 1.5. Where mixtures of block copolymers are used, n will be the weighted average of n for each contributing copolymers, with the averaging based on the weight portions of the component copolymers. When copolymers other than polyethylene oxide-polypropylene oxide copolymers are used, similar approaches can be developed to relate the hydrophobic/hydrophilic properties of one member of the class of polymers to the properties of another member of the class.

The polynucleotide compositions of the invention can be administered orally, topically, rectally, vaginally, by pulmonary route by use of an aerosol, or parenterally, i.e. intramuscularly, subcutaneously, intraperitoneallly or intravenously. The polynucleotide compositions can be administered alone, or it can be combined with a pharmaceutically-acceptable carrier or excipient according to standard pharmaceutical practice. For the oral mode of administration, the polynucleotide compositions can be used in the form of tablets, capsules, lozenges, troches, powders, syrups, elixirs, aqueous solutions and suspensions, and the like. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral

The NIH 3T3 cells were grown in monolayer culture at 37°C under 5% CO_2, utilizing a DMEM medium containing 2 mM glutamine and 10% fetal calf serum ("FCS"). Cells were grown in monolayer culture were scraped and prepared for the transaction process by washing three times with fresh medium.

Alloquots of washed cells that were to be transformed by the method of the invention were suspended at a concentration of 10^6 cells/ml in Pluronic A transfecting medium. The suspended cells were incubated for 2 hours at 37°C and under 5% CO_2. The cells were then washed with fresh medium and re-plated.

Alloquots of cells that were to be transfected by calcium phosphate precipitation were transfected as recommended by Promega of Madison, Wisconsin, in their manuscript *Profection Mammalian Transfection Systems*, Technical Manual, 1990. Specifically, pβ-Gal was mixed with 0.25 M $CaCl_2$. The mixture was mixed with an equal volume of 2x HBS (Hanks Buffer Salt, available from GIBCO, Grand Island, NY) to create a mixture containing 1μg/mL pβ-Gal. The opaque mixture was incubated at room temperature for 10 minutes and then applied to the cells. The suspended cells were incubated for 2 hours at 37°C and under 5% CO_2. The cells were then washed with fresh medium and re-plated.

The replated cells were incubated for 48 hours in DMEM medium containing 10% FCS. During the incubation, the medium was replaced with fresh medium at 16 hours. After the 48 hour incubation, the cells for each incubation were collected by scrapping, washed with PBS, and resuspended in 100 μl of 0.2 M Tris-HCL (pH 7.4). The cells were lysed with several freeze/thaw cycles, and centrifuged at an excess of 6,000 x/g. 50 μl of supernate was removed from each lysate tube and mixed with 50 μl of a solution of 0.1 mM 4-methyl-umbelliferril-β-D-galactopiraniside (the substrate), 0.1 M sodium phosphate (pH 7.4). Each mixture was incubated for 20 min. at 37°C to allow any β-galactosidase present to act on the substrate. 50 μl of 0.4 M glycine, pH 10.5, was added to terminate the β-galactosidase reaction. β-galactosidase activity

After treatment with Pluronic B transfecting solution or calcium phosphate, the cells were washed 5-6 times with fresh medium. They were then incubated in DMEM containing 10% FCS for 48 hours at 37°C, under 5% CO_2. After the first 16 hours of this incubation, the medium was replaced. After the incubation, the cells were washed with PBS, released from their plates by trypsinization, and again washed with PBS. β-Galactosidase was measured as described for Example 1. The results were as follows:

Treatment	Relative β-galactosidase activity ± SEM (n = 4)
Pluronic B	910 ± 45
Calcium Phosphate Precipitation	81 ± 17

Example 3 - Transfection Experiments - First Embodiment Complex

In these experiments, transfection efficiencies with Chinese hamster ovary (CHO) cells were examined. The polynucleotic component of the polynucleotic complex was pβ-Gal. The polycation component comprised pEVP-Br. The block copolymer comprised an octablock copolymer formula (XVII), wherein i was equal to 10 and j was equal to 12 (hereinafter "Pluronic C" available from BASF). A Pluronic C transfecting solution of 1 μg/ml pβ-Gal, 4μg/ml pEVP-Br, and 1% (w/v) Pluronic C was prepared as in Example 1. The ratio of basic groups to nucleotide phosphates was 10. The weight ratio of Pluronic C to pβ-Gal was 10^3. The transfection protocol was the same as that used in Example 2. The results were as follows:

Treatment	Relative β-galactosidase activity ± SEM (n = 4)
Pluronic B	910 ± 45
Calcium Phosphate Precipitation	81 ± 17

Example 4 - Bacterial Transformation - Second Embodiment Complex

In these experiments, transformation efficiencies using the MC5 strain of *Bacillus subtilis* were examined. The polynucleotide component of the

digestion times, was determined by electrophoresis in a polyacrylamide gel. See Kabanov et. al., *Biopolymers*, 31:1437-1443 (1991). The results were as follows:

Time of Incubation	Circular DNA (% of initial)	
	Complex	Free DNA
0	100	100
5	100	20
10	100	8
30	100	4
60	100	1
180	100	0
600	100	0

Example 6 - Oligonucleotide Stabilization

For this example, a complex containing an oligonucleotide complementary to the transcription initiation site of the HIV-1 tat gene ("anti-tat", comprising GGCTCCATTTCTTGCTC) was prepared using the diblock copolymer of formula (XIX) (polyoxyethylene-poly(L-alanine-L-lysine), wherein i is 44 and j is 8). The oligonucleotide complex was prepared in PBS Buffer (pH 7.0) at a concentration of 0.75 $OD_{260}/\mu l$ oligonucleotide. The ratio of polycation imino and amino groups to polynucleotide phosphate groups was about 50. The mixture was incubated for one hour at room temperature to allow for the formation of the complex. Then, the complex was purified by gel filtration chromatography on Sephadex G-25 using 0.05 M NaCl as the eluent. The resulting solution of complex exhibited a concentration of 0.11 $OD_{260}/\mu l$ of oligonucleotide. A comparable solution of uncomplex oligonucleotide was prepared. An alloquot of murine blood plasma (10 μl) was mixed with an equal volume of oligonucleotide complex solution or a solution of free oligonucleotide. Samples were incubated at 37° C for various time periods. To stop the reaction of the oligonucleotides with enzymes in the plasma, the samples were diluted with water and extracted with a water-saturated mixture of phenol : chloroform (1:1). The aqueous phase of the extraction was isolated, and the oligonucleotide therein was precipitated with 3%

cells. SKVLB cells are multi-drug resistant cells derived from a ovarian cancer cell line. The MDR1 gene has been identified as responsible for the multi-drug resistance in SKVLB cells. Endicott and Ling, *Ann. Rev. Biochem.*, 58:137 (1989). In particular, the efficiency of the anti-MDR oligonucleotide in the polynucleotide complex of the invention and when in the free state was compared. As controls, the free and complexed form of the anti-tat oligonucleotide described above were also used. The polynucleotide complexes were formed with the diblock copolymer of formula (XX) (polyoxyethylene-polypropyleneimine/butyleneimine, where i was 44 and j was 9-10). The complexes were prepared by the procedures described in Example 6. The oligonucleotide concentration in the complex or in the free state was 0.17 $OD_{260}/\mu l$. The copolymer was present in the concentration sufficient to define a ratio of polycation block imino and amino groups to oligonucleotide phosphate groups of 10.

The SKVLB cells were incubated for 3 days at 37° C under 5% CO_2 in the presence of free or complexed oligonucleotide (at a concentration of 20μM based on oligonucleotide content). Fresh media including free or complexed oligonucleotide was added every 12 hours.

The daunomycin cytotoxicity (IC_{50}) with respect to the cells treated as described above was measured using the method of Alley et. al., *Cancer Res.*, 48:589-601. The results were as follows:

Treatment of Cells	Daunomycin IC_{50} (ng/ml) (n=4)
Control (untreated cells)	8.0
Anti-MDR Complex	0.3
Anti-tat Complex	8.2
Free Anti-MDR	2.1
Free Anti-tat	7.9

Example 9 - Antisense Oligonucleotide Designed to Inhibit Herpes Virus

This experiment utilized a 12-chain oligonucleotide, which had been covalently modified at its 5´ end with undecylphosphate substituent and at is 3´

Example 10 - Antisense Oligonucleotide Designed to Inhibit Herpes Virus

Unless otherwise noted, this example utilized the same procedures as were utilized in Example 9. The cells utilized were BHK cells, a Chinese hamster kidney cell line. When the complexed form of the oligonucleotides was used, the complex was formed with the diblock copolymer of formula (XVII) (polyoxyethylene-poly-L-lysine, wherein i was 44 and j was 30), using the procedure described in Example 6. The concentration of the stock solution of complex was 0.09 $OD_{260}/\mu l$. The ratio of polycation block imino and amino groups to oligonucleotide phosphates was 10. The oligonucleotides, in complexed or free form, were applied to the cells at a concentration of 3.0 μM. The results were as follows:

Treatment of cells	HSV-1 infectious titre (CPE_{50}/ml) n = 7
Control (untreated infected cells)	$10(\pm 3)x10^3$
Anti-HSV complex	$8(\pm 6)$
Anti-influenza complex	$13(\pm 4)x10^3$
Free Anti-HSV	$50(\pm 14)x10^2$
Free Anti-influenza	$9(\pm 2)x10^3$

Example 11 - In Vivo Inhibition of HSV

Polynucleotide complexes between the block copolymer of formula (XVII) (polyoxyethylene-poly-L-lysine, wherein i was 44 and j was 30) and the Anti-HSV and Anti-Influenza oligonucleotides were formed using the methods outlined in Example 9. The concentration of the stock solutions of complexes was 0.9 $OD_{260}/\mu l$. The ratio of polycation block imino and amino groups to oligonucleotide phosphates was 10.

Inbred white mice (body weight: 6 to 7 g) were infected with HSV-1 (strain C1 from *Belorussian Res. Inst. of Epidemiol. & Microbiol.*, Minsk) by intraperitoneal injection of 30 μl of a virus suspension (titre: 10^{-7} LD_{50}/ml). Either Anti-HSV complex, Anti-Influenza complex, free Anti-HSV or free Anti-Influenza were injected (10 μl) into the tail vein of a given mouse at each of 2, 12, 24, 48 or 72 hours post-infection. The results were as follows:

Time after injection (min)	Plasma levels (% of injected dose)		Liver levels (% of injected dose)	
	Anti-HIV Conjugate	Free Anti-HIV	Prep. A	Prep. B
0	100	100	0	0
5	95	58	3	7
10	91	40	5	19
15	84	33	7	26
20	79	27	9	30
30	75	20	10	35

Example 13 - Cationic Block Copolymer Synthesis

1,4-dibromobutane (5.4 g, 25 mmoles, from Aldrich Co., Milwaukee, WI) was added to a solution of N-(3-aminiopropyl)-1,3-propanediamine (6.55 g, 50 mmoles, from Aldrich Co.) dissolved in 100 ml of 1,4-dioxane. This reaction mixture was stirred at 20°C for 16 h. The product of this reaction spontaneously precipitates from solution as the hydrobromide salt. This precipitated first intermediate was collected and twice dried by rota-evaporation from a solution of 10% triethylamine in methanol. This evaporation procedure was effective to remove substantial amounts of the bromide salt. The first intermediate was dissolved in 50 ml of 1,4-dioxane and reacted with 2.7 g (12.5 mmoles) of 1,4-dibromobutane. Again, the reaction proceeded for 16 h at 20°C, and the resulting second intermediate was recovered and dried as above. The second intermediate was neutralized with acetic acid to a pH of 7-8 and purified by gel filtration on Sephadex G-25, using an aqueous eluent. Three major polymine fractions were obtained, having apparent molecular weights of 1060, 700 and 500, respectively.

Poly(oxyethyleneglycol) (1.5g, M.W. 1500, from Fluka) was dissolved in 8 ml of 1,4-dioxane and reacted with 0.17 g (1 mmole) of N,N'-carbonylimidazole (Aldrich Co.) at 20°C for 3 h. The reaction mixture was divided into two parts. Each part was mixed with 4 ml of a 10% (w/v) solution of either the 1060 or 700 MW polyimine fraction, which solution further contained 0.01N NaOH. The

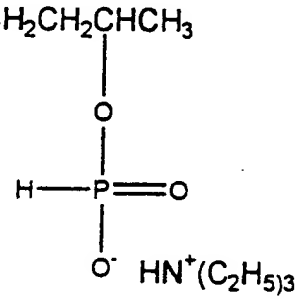
Internat., 25:767 (1991); Vinogradov et al., *BBRC*, 203:959 (1994). "Pluronic A", a block copolymer of formula (XIV)(x=25, y+38, z=25) was similarly oxidized to create terminal aldehydes. The amine derivative (1 mg) was dissolved in 100 μl of 0.1 M borate buffer (pH 9.0) and mixed with 2 mg of the Pluronic A derivative. 1.5 mg of sodium cyanoborohydride was added to the mixture to reduce the Schiff's bases formed between the amine and aldehyde groups. This reaction was allowed to proceed for 12 hours at 4°C. The polymeric product of this reaction was isolated by gel filtration chromatography on Sephadex LH-20, utilizing 90% aqueous isopropanol as the eluent. The conjugate so obtained is referred to hereinafter as "Oligo A Conjugate."

Example 17- The Effect of Oligo A Conjugate on Virus Production

Oligo A and Oligo A Conjugate were separately dissolved in RPMI 1640 medium (ICN Biomedicals Inc., Costa Mesa, CA) to a final concentration of 0.2 mM (based on oligonucleotide absorbance). These stock solutions were then filtered through 0.22 μm filters to remove any possible bacterial or fungal contamination.

Monolayers of Vero cells were incubated for 1 hour at 37°C in serum-free RPMI 1640 together with various concentrations of Oligo A or Oligo A Conjugate. The monolayers, while still exposed to oligonucleotides, were then infected with 1 plaque forming unit per cultured cell of HSV-1, strain L2 (from the Museum of Virus Strains of the D.I. Ivanovskii Institute of Virology, Russian Academy of Sciences, Russian Federation). This infection method has been described by Vinogradov et al., *BBRC*, 203:959 (1994). After 8 hours of exposure to virus and oligonucleotides, the medium on the cells was replaced with fresh medium containing 10% FCS. Medium from the cells was collected at 22 and 39 hours after the infective incubation, and the virus titer in the collected medium was

1,4-dioxane were added to the diisopropyethylamine solution in small portions under an inert, argon atmosphere. The reaction mixture was incubated during 1 hour at 20°C. The reaction was monitored by the thin layer chromatography as described above. The Rf of the product was 0.05. 10 mls of water were added to the reaction mixture. After 30 min., the solvent was evaporated. The product was dissolved in 100 ml of chloroform and the solution obtained was washed stepwise with (1) 100 ml of 8% aqueous solution of the sodium bicarbonate, (2) 100 ml of 0.2 M triethyammoniumacelate solution (pH 7.2), and (3) 100 ml of water. The organic solvent was evaporated and the oily remainder, containing the phosphonate monomer was purified by chromatography on silicagel column, using stepwise gradient of (1) chloroform, (2) 3% methanol in chloroform and (3) 6% methanol in chloroform. The yield of the monomer was 4.1 g (=7.3 mmol, 63%). The product, having structure



wherein DMT represents a dimethoxytrityl group, can be termed "Phosphonate Monomer A."

Example 19 - Synthesis of Polycation BDP

A 0.05 M solution of the phosphonate Monomer A in anhydrous pyridine:acetonitrile mixture (1:1) was placed in the position 6 of the DNA-synthesator (model 380-B02, Applied Biosystems, CA). A 2% solution of adamantoilchloride (Sigma) in the mixture acetonitrile:pyridine (95:5) was used as a condensing agent. The synthesis was conducted using the program modified for an H-phosphonate cycle (Sinha and Striepeke In: *Oligonucleotides and Analogues: A Practical Approach*, Eckstein Ed. IRL Press, Oxford, New York-Tokyo, p. 185, 1991) and the DMT-group was preserved after the synthesis was complete. Adenosine (4 μmoles) immobilized on a standard CPG-500 solid

BDP concentration in cell medium, μM	Infectious titer, PFU/ml
0 (polycation-free virus)	2×10^7
1	2×10^7
2	2×10^7
4	1×10^8
8	7×10^8
15	2×10^9

What is claimed:

1. A polynucleotide composition comprising:

(a) a polynucleotide or a polynucleotide derivative; and

(b) up to about 15% of (w/v) a polyether block copolymer comprising an A-type polymeric segment comprising a linear polymeric segment, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or less and have molecular weight contributions between about 30 and about 500, and a B-type polymeric segment comprising a linear polymeric segment, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, and wherein at least about 80% of the linkages joining the repeating units for each of the polymeric segments comprise an ether linkage.

2. The polynucleotide composition of claim 1 wherein said block copolymer is selected from the group of block copolymers consisting of

A-B-A', A-B, -B-A-B', and $L(R^1)(R^2)(R^3)(R^4)$

(I) (II) (III) (IV)

wherein A and A' are A-type linear polymeric segments, wherein B and B' are B-type linear polymeric segments, and wherein R^1, R^2, R^3 and R^4 are (1) block copolymers of formulas (I), (II) or (III) or (2) hydrogen and L is a linking group, with the proviso that no more than two of R^1, R^2, R^3 or R^4 shall be hydrogen, and mixtures thereof.

3. The polynucleotide composition of claim 1 wherein 90% of the linkages joining the repeating units for each said polymeric segment comprise ether linkages.

4. The polynucleotide composition of claim 1 wherein the repeating units for each said polymeric segment have molecular weight between about 30 and about 100.

5. The polynucleotide composition of claim 4 wherein 90% of the linkages joining the repeating units for each said polymeric segment comprise ether linkages.

(3) a heterocyclic group, having 3-8 ring atoms, which can include heterocycloalkyl or heteroaromatic groups, which can include from 1 to 4 heteroatoms selected from the group consisting of oxygen, nitrogen, sulfur and mixtures thereto, and which can include alkyl having 1-6 carbon atoms, alkoxy having 1-6 carbon atoms, alkylamino having 1-6 carbon atoms, dialkylamino wherein each alkyl independently has 1-6 carbon atoms, amino, sulfonyl, hydroxy, carboxy, fluoro or chloro substituents.

10. The polynucleotide composition of claim 9 wherein all of the repeating units that comprise blocks B or B' have a Hansch-Leo fragmental constants of about -.30 or more.

11. The polynucleotide composition of claim 10 wherein all of the repeating units that comprise blocks A or A' have a Hansch-Leo fragmental constants of about -0.4 or less.

12. A polynucleotide composition comprising:

(a) a polynucleotide or a polynucleotide derivative; and

(b) a copolymer having a polyether segment and a polycation segment, wherein the polyether segment comprises an A-type polymeric segment comprising a linear polymeric segment, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or less and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the linkages joining the repeating units of the polyether segment comprise an ether linkage, and wherein the polycation segment comprises a plurality of cationic repeating units.

13. The polynucleotide composition of claim 12 wherein said copolymer comprises a polymer of formula:

B-A-R, A-R, A-R-A', and R-A-R',

(V) (VI) (VII) (VIII)

wherein A and A' are A-type linear polymeric segments, wherein B and B' are linear polymeric segments, the monomers of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the inter-monomer linkages for each said polymeric segment comprise

wherein pN represents a polynucleotide having 5′ to 3′ orientation, wherein A and A′ are A-type linear polymeric segments, wherein B and B′ are B-type linear polyether segments, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the linkages joining the repeating units of the polyether segment comprise ether linkages, and wherein R and R′ are polymeric segments comprising a plurality of cationic repeating units.

20. The polynucleotide composition of claim 18 wherein the polynucleotide derivative comprises at least a second polyether segments, which can comprise an A-type polyether segment or a B-type polyether segment comprising a linear polymeric segment, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the linkages joining the repeating units of the B-type polyether segment comprise ether linkages.

21. A polycationic polymer comprising a block copolymer comprising a polyether segment and a polycation segment, wherein said polyether segment comprises (a) at least one of (1) an A-type polymeric segment comprising a linear polymeric segment, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or less and have molecular weight contributions between about 30 and about 500, or (2) a B-type polymeric segment comprising a linear polymeric segment, the repeating units of which contribute an average Hansch-Leo fragmental constant of about -0.4 or more and have molecular weight contributions between about 30 and about 500, wherein at least about 80% of the linkages joining the repeating units for either of the polymeric segments comprise an ether linkage, and (b) a polycationic segment comprising a plurality of cationic repeating units of formula -NH-R^0- wherein R^0 is a straight chain aliphatic group of 2 to 6 carbon atoms, which may be substituted.

22. The polycationic polymer comprising a block copolymer of claim 21 comprising a block copolymer according to formulas:

INTERNATIONAL SEARCH REPORT

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PCT/US95/13800

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/00
US CL :514/44; 536/22.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS MEDLINE BIOSIS CAPLUS WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,047,236 (HUNTER et al.) 10 September 1991, see entire document.	1-22
Y	Journal of Controlled Release, Volume 28, issued 1994, KABANOV et al., "New approaches to targeting bioactive compounds", pages 15-35, see entire document.	1-22
Y	Nucleic Acids Research, Volume 22, Number 22, issued 1994, JASCHKE et al., "Synthesis and properties of oligodeoxyribonucleotide-polyethylene glycol conjugates", pages 4810-4817, see entire ddocument.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search	Date of mailing of the international search report
10 MARCH 1996	22 MAR 1996
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